Comparison of three blood transfusion guidelines applied to 31 feline donors to minimise the risk of transfusion transmissible infections.

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Abstract

Objectives The increased demand for animal blood transfusions creates the need for an adequate number of donors. At the same time, a high level of blood safety must be guaranteed and different guidelines (GLs) deal with this topic. The aim of this study was to evaluate the appropriateness of different GLs in preventing transfusion-transmissible infections (TTI) in Italian feline blood donors.

Methods Blood samples were collected from 31 cats enrolled as blood donors by the owners' voluntary choice over a period of approximately 1 year. Possible risk factors for TTI were recorded. Based on Italian, European and American GLs, specific TTI, including haemoplasmas, feline leukemia virus (FeLV), feline immunodeficiency virus (FIV), *Anaplasma phagocytophilum, Ehrlichia* spp., *Bartonella* spp., *Babesia* spp., *Theileria* spp., *Cytauxzoon* spp., *Leishmania donovani* sensu lato and feline coronavirus (FCoV) were screened. Rapid antigen and serological tests and biomolecular investigations (PCR) were used. Several PCR protocols for haemoplasma and FeLV DNA were compared to each other.

Results The presence of at least one recognized risk factor for TTI was reported in all cats. Results for FIV and FeLV infections were negative using rapid tests, whereas 5 (16.1%) cats were positive for FCoV antibodies. Four (12.9%) cats were PCR positive for haemoplasma DNA and 1 (3.2%) for FeLV provirus, the latter being positive only using the most sensitive PCR protocol applied. Other TTI were not detected using PCR.

Conclusion and relevance Blood safety increases by combining the recommendations of different GLs. To reduce the risk of TTI, sensitive tests are needed and the choice of the best protocol is a critical step in improving blood safety. The cost and time of the screening procedures may be reduced if appropriate tests are selected. To this end, the GLs should include appropriate recruitment protocols and questionnaire-based risk profiles to identify suitable donors.

Introduction

Recently, the increase of the indications for the veterinary blood transfusion and its routine use in the veterinary practice caused a rise in the demand for animal donors, At the same time, a high level of blood safety must be guaranteed to perform this procedure.

Transfusion-transmitted infections (TTI) from apparently healthy and asymptomatic blood donors represent a well-known threat in blood transfusion, in addition to other adverse events.[1-4] Therefore, the identification of risk factors and characteristics of "low risk" blood donors are very important for increasing blood safety. Appropriate recruitment strategies could also reduce infection risks. In human medicine, the World Health Organization (WHO) identified regular voluntary non-remunerated donors as those with the lowest risk for TTI.[5] However, this model cannot be used because different situations and settings exist in veterinary medicine. In cats, different prevalences of microorganisms were found based on the source of the feline donors: a previous study found that laboratory-reared cats and cats housed indoor with no history of flea or tick infestation were ideal blood donors.[6]

Moreover, strategies and procedures ensuring blood safety for TTI change in different settings.[7] For humans, it is well known that each country has to address specific issues or constraints that influence the safety of its blood supply, including the incidence and

prevalence of TTI, the structure and level of development of blood transfusion services, and the economic resources available.[5]

There are several guidelines (GLs) for testing protocols in veterinary medicine. GLs are generally developed according to the circumstances and needs of each country. No single GL can ensure absolute blood safety.[5] Both American and European GLs aim to standardize veterinary blood transfusion procedures and to guarantee blood safety.[8-10] Additional GLs have been published in Italy to regulate veterinary blood transfusions in dogs, cats and horses in this country.[11-12] However, all these GLs differ in some aspects, such as the microorganism to be screened or the screening methods.[8-10]

The aims of this research were i) to evaluate the differences among existing GLs, and ii) to perform a preliminary investigation on a selected population of feline blood donors from an Italian blood bank, evaluating how blood safety can be improved using tests recommended by the different GLs.

Materials and methods

Comparison of International and National GLs

The main existing GLs for veterinary blood transfusions were compared.[8-12] The European and American GLs were written by a panel of experts, according to evidence-based medicine, even if the level of evidence for each topic was not always explicitly

87 declared.[8-10] The Italian GL for animal transfusion was written by a panel of experts
88 commissioned by the Italian Ministry of Health.[11,12]
89
90 **Feline blood donor selection**
91 During the January 2014-January 2015 period, blood samples were collected from cats
92 enrolled as blood donors at the Blood Bank of the Veterinary Teaching Hospital of the
93 University of Perugia. Cats were enrolled using the criteria of suitability indicated in the
94 Italian GL:[11,12] body weight ≥5 kg, age 2-8 years, docile character, regularly vaccinated
95 with core vaccines (feline calicivirus, feline herpesvirus, feline parvovirus) and two
96 non-core vaccines (*Chlamydia felis*, formerly *Chlamydophila felis*, and feline leukemia
97 virus, FeLV). Blood donors enrolled in cases of life-threatening emergencies, for which
98 the national GL recommends fast and restricted screening limited to feline
99 immunodeficiency virus (FIV), FeLV and *Mycoplasma haemofelis*, were excluded from
100 the study.
101 The owners of cat blood donors were asked for the following information: complete
102 history, blood collection date, type of owner (staff or client), identification of the
103 animal, age, gender, weight, breed, origin (adoption from breed or stray cat or purchase
104 at a pet shop), type of housing (indoor *vs* outdoor or mixed), cohabitation with other
105 cats, ectoparasite treatment and frequency, coat (long or short hair), and laboratory test

results. Travel history was not systematically investigated because initially not included in the information required.

For each potential donor, before the collection of blood, a careful physical examination, was performed, with particular emphasis on the presence of fleas or ticks, and biological samples were collected by a single operator for clinicopathologic screening, consisting of a complete blood count, typing of blood group, serum chemistry, urinalysis, and faecal examination.

Blood donation was a voluntary choice by the owners, who signed a written consent form to authorize blood collection and storage and the use of samples and data for scientific purposes. Therefore, based on the current regulations of our institutions, the formal approval of the ethical committee was not needed for this study.

Sample collection

One ml of blood from each donor was placed into 2 anticoagulated tubes containing sodium-citrate or EDTA (Ethylene-diamine-tetra-acetic acid) to obtain buffy coat and plasma, respectively. Three ml of blood were placed in a plain Vacutainer tube (Becton Dickinson, Milan, Italy) to obtain serum by centrifugation (1000 x*g* for 10 min). An aliquot of each sample was stored at -80 °C for further investigations.

TTI screening

126 Serum samples were screened by rapid test using an ELISA for FeLV antigen and FIV
127 antibodies (SNAP FIV/FeLV Combo test, IDEXX Laboratories) and an
128 immunochromatographic method for feline coronavirus (FCoV) antibodies (FASTest
129 FIP, MegaCor Diagnostik).
130 For each cat a Romanowsky-stained blood smear was microscopically examined to
131 assess the presence of morphologically detectable microorganisms, with attention given
132 in particular to haemoplasmas.
133 DNA and RNA were extracted from 200 µL of both buffy coat and whole blood
134 samples according to validated protocols (Supplementary Table),[13-25] using a
135 commercial kit for viral RNA and DNA and bacterial DNA (QIAamp cador Pathogen
136 Mini Kit, Qiagen, Milan, Italy), in accordance with the manufacturer's instructions. The
137 concentration and purity of the extracted nucleic acids were quantified using a
138 NanoDrop® spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Milan,
139 Italy).
140 Previously published PCR assays, used for the diagnostic activities in the laboratory that
141 performed the tests, were applied to detect the infectious agents (Supplementary
142 Table).[13-25] Several published PCR protocols were performed in the case of
143 haemoplasmas[13-15] and FeLV[18-20] to compare the sensitivity of the assays. In this case,
144 10-fold dilutions of positive samples were used.

145 A PCR targeting the 18S ribosomal RNA gene was used as internal control to rule out
146 possible PCR inhibitors in the samples.
147 With regards to FeLV, the PCR product of the expected size was purified with an
148 extraction kit (Qiaquick PCR purification kit, Qiagen) and directly sequenced on both
149 strands with the specific primers previously described,[20] using a DNA analyzer (ABI
150 3730, Applied Biosystems) capillary sequencer (Bio-Fab Research srl). The sequences
151 were assembled and aligned using BioEdit software[26] and sequence similarities were
152 assessed by comparison with the sequences deposited in GenBank using the BLAST
153 software.[27]
154
155 **Statistical analysis**
156 Chi-square, with Yates's correction, and Fisher's exact test were used to compare the
157 proportions of positive and negative samples, stratified for the data of the animals at the
158 time of the visit (staff- or client-owner, age, gender, breed, origin, type of housing,
159 cohabitation with other cats, ectoparasite treatment and frequency, kind of coat), as
160 most appropriate. Age was analysed by grouping the animals into age categories testing
161 a cut-off $\leq$ 3 years. A P value <0.05 was considered statistically significant. EpiInfo[28]
162 and OpenEpi[29] were used for analysis.
163
164 **Results**

Comparison of International and National GLs

Different aspects concerning criteria of suitability, selection and TTI screening for blood donors are reported in the GLs.[8-12] However, all GLs consider FIV, FeLV, and *Mycoplasma haemofelis* as the minimum essential level of screening (Tables 1 and 2). Based on the epidemiological situation of the individual country [14,30-37] and as suggested by all GLs, Italian GLs were partially integrated with European and American GLs for increasing blood safety. Accordingly, further TTI were investigated: *Candidatus* Mycoplasma haemominutum, *Candidatus* Mycoplasma turicensis, *Anaplasmataceae* family (*Anaplasma phagocytophilum* and *Ehrlichia* spp.), *Bartonella* spp., *Babesia* spp., *Theileria* spp., *Cytauxzoon* spp., *Leishmania donovani* sensu lato, and FCoV. In addition, biomolecular methods were added to the procedures recommended by the Italian GL.[12]

Feline blood donor profiles

Thirty-one cats from 18 different owners were included in the study. Six (19.4%) were female (3 neutered) and 25 (80.6%) male (15 neutered). The mean age was 4 years (median 5, range 2-8) and the mean weight 6.23 kg (median 6, range 5-9). Twenty-three (74.2%) were domestic shorthair, whereas 8 (25.8%) were Maine Coon. Twenty cats (64.5%) were client-owned and 11 (35.5%) staff-owned, including students of the veterinary medicine course. Based on the answers received, 22 (71%) cats were adopted

185 stray kittens, whereas 9 (29%) were purebred cats. Thirteen (44.8%) cats lived indoors,
186 whereas 16 (55.2%) had access to the outdoors. Twenty-six (83.8%) lived with other
187 cats. Only 2 owners performed regular ectoparasite treatments, whereas 25 others
188 (89.3%) administered the treatments mainly in warm periods, and 1 never administered
189 ectoparasite treatments. Thirteen cats (46.4%) were long-haired, and 15 (53.6%) short-
190 haired. In three cases the recording of the answers missed to collect information on the
191 kind of coat and treatment, and in two cases the type of housing.
192 At least 5 owners travelled with their cats in different areas of southern-central Italy.
193 No cats had recent illnesses. Physical examination and clinicopathologic screenings
194 were unremarkable, with all the parameters within the standard reference intervals, and
195 cats were considered eligible for blood donation.
196
197 **TTI screening**
198 All cats were negative using FIV and FeLV rapid tests, whereas 5 (16.1%) were
199 positive for FCoV antibodies.
200 Blood smears did not reveal morphologically detectable microorganisms.
201 Cats were negative using PCR for *Anaplasmataceae* (*Anaplasma* spp. and *Ehrlichia*
202 spp.), *Bartonella* spp., *Babesia* spp., *Theileria* spp., *Cytauxzoon* spp., *Leishmania*
203 *donovani* sensu lato and FCoV, whereas haemoplasma DNA was detected in 4 cats
204 (12.9%) using all the PCR protocols targeting the 16S rRNA gene.[13-15] However, the

205 nested PCR[14] was found to be 10-fold more sensitive than the other PCR protocol
206 used.[13,15] Further specific tests[16,17] identified three cats as infected with *Candidatus*
207 Mycoplasma haemominutum and one with *Mycoplasma haemofelis*. The latter cat was
208 also PCR positive for FeLV provirus using the specific nested PCR protocol,[19] but not
209 with the others protocols used to detect FeLV DNA.[18,20] The specific nested protocol[19]
210 was at least 10-fold more sensitive than the others.[18,20] Sequencing of the positive FeLV
211 PCR product shared 98%-100% identity with the homologous FeLV region LTR
212 sequences reported in GenBank (accession nos. AY374218, L25630, M18248,
213 KP728112).
214
215 **Statistical analysis**
216 No statistical association with positivity to the screened microorganisms and the
217 characteristics of the cats was found.
218
219 **Discussion**
220 In this study, Italian GLs were applied to 31 feline candidate blood donors enrolled over
221 a 1-year period. According to these GLs, these cats were fully eligible as blood donors.
222 However, based on donor selection criteria or recommendations of other GLs, blood
223 safety could not be completely guaranteed. Indeed, the enrolled cats, although clinically

224 healthy, had risk factors for harbouring TTI and resulted also positive for some
225 microorganisms included in the other GLs.
226 All 31 cats were negative using rapid tests for FIV and FeLV. One cat was positive for
227 FeLV provirus, but only when using the most sensitive PCR protocol. This cat should
228 be excluded from the donation program, considering that FeLV provirus carriers testing
229 negative for the p27 antigen may transmit FeLV infection to a naïve recipient via blood
230 transfusion.[38] These results show that the Italian GL would have missed this infected cat
231 because, as opposed to the other GLs,[9,10,39] PCR is not even recommended for detecting
232 proviral FeLV. Furthermore, the results also raised the issue that the same animal may
233 be classified eligible or ineligible as a blood donor based on the different PCR assay
234 that is applied.
235 Blood smears negative for haemoplasmas cannot exclude infection. The sensitivity of
236 microscopic analysis is too low to detect haemoplasmas in chronically asymptomatic
237 cats and does not differentiate the Mycoplasma species.[40,41] Using sensitive PCR
238 protocols, as suggested by European and American GLs and previous studies,[2,3,8,10]
239 12.9% of cats were positive, with a prevalence comparable with that of previous studies
240 on blood donors.[37,40,42] This confirms the endemicity of haemoplasma in clinically
241 healthy cats and the high probability of finding a positive cat even in a limited number
242 of samples.[6,40] All the PCR protocols[13-15] identified the positive cats, but the nested
243 PCR[14] was 10-fold more sensitive than the other protocols. This protocol should be

244 preferred for its higher sensitivity, since fluctuations in the number of circulating
245 haemoplasmas may lead to false negative results using less sensitive protocols.[41] For the
246 same reason, TTI screening should always be performed on the same blood collected for
247 donation: negative results for blood samples collected even few days before the
248 donation cannot rule out the presence of hemoplasmas in subsequent blood samples
249 used for transfusion because of the fluctuation in bacteremia. Moreover, considering
250 that the highest risk of haemoplasma transmission by blood was found within one week
251 from its collection[2] and that the risk in feline blood donation was recently defined non-
252 negligible,[37] sensitive PCR tests should be recommended.
253 *Mycoplasma haemofelis*, the only species included in the Italian and updated American
254 GLs, was detected in only one cat, whereas three cats were positive for *Candidatus*
255 Mycoplasma haemominutum. The Italian GL recommends temporarily excluding cats
256 infected by *Mycoplasma haemofelis* from the donation program until PCR results are
257 negative, whereas American GLs exclude them permanently, assuming that these cats
258 may be chronic carriers of Mycoplasma.[41,43] The identification of haemoplasma species
259 is considered optional by the American GL.[9] However, four haemoplasma species are
260 recognized in cats, and their pathogenic significance is debated:[40,41,44] hence, the
261 identification of Mycoplasma species, although time-consuming, may be recommended.
262 Although not currently recommended by all GLs, the screening of FCoV antibodies
263 using rapid tests, as is required by the Italian GL, showed a low prevalence of infection

(16.1%) compared to previous studies in Italian populations.[35,45] Furthermore, a specific nested PCR assay demonstrated that blood samples were negative for FCoV. However, the European GL[10] and a recent study[46] advise that seropositive cats should be excluded from the donor program because it is possible that passively transferred anti-FCoV antibodies could endanger the recipient, if infected, even if no evident risk of transmission of FCoV via blood has been demonstrated:[46,47,48,49] and no reports of transmission following blood transfusion have been described until now. On the other hand, there is no evidence that seropositive cats will develop FIP.[9,10] With the shortage of donor cats already available, the exclusion of FCoV seropositive cats is likely unnecessary and could cause a relevant reduction in the number of blood donor cats. Moreover, considering that transitory viremia is possible even in seronegative cats,[45,50] FCoV RNA screening of blood collected for donation could be appropriate.

The comparison of analytical sensitivities of the different protocols was limited to haemoplasmas and FeLV PCR assays. However, this approach would have been appropriate also for the other tests. Therefore validated and common protocols could be described and advised by GLs in order to guarantee the same level of accuracy in detecting TTI blood donors everywhere, as is done for notifiable diseases.[51] As in people, however, test accuracy may be time-consuming and expensive. Otherwise, a lower level of risk assessment should be accepted by the owner of the recipient by

283 written consent, such as in the case of emergency transfusions, as is currently indicated
284 by all GLs.[9,10,12]
285 Accordingly, additional appropriate strategies for selecting "low risk donors" should be
286 applied to reduce the cost and time of screenings and to guarantee a high level of blood
287 safety. For example, the use of a questionnaire to identify suitable donors could be an
288 inexpensive and useful tool. As is already the practice in human medicine, recently both
289 American and European GLs provided different questionnaires to determine the risk
290 profile.[9,10] However, even if the questionnaire was not available at the time of enrolment
291 in the study, the owners in the current study were asked specific questions and the
292 profiles of the donor candidates showed that they had risk factors for harbouring TTI.
293 All 31 Italian donor cats had at least one TTI-risk characteristic or behavior, such as
294 access to the outdoors, for which American GL specifically recommends repeated
295 testing,[9] or the irregular ectoparasite treatment performed by 89.3% of the owners. All
296 GLs advise regular ectoparasite treatments, but the frequency of treatments is not
297 specified. Monthly ectoparasite prophylaxis was suggested by other studies.[3,7] The
298 owners should be encouraged to carry out ectoparasite prophylaxis correctly,
299 considering the relevance of this practice in the control of a wide range of vector-borne
300 diseases. Although travel history was not systematically investigated, at least five
301 owners travelled with their cats in different areas of southern-central Italy, that are at
302 risk of some infectious diseases and therefore should be considered at high risk for

harbouring TTI.[33,34] Unfortunately, the small number of cats in this study limited the statistical power of this study. A higher number of animals could improve the identification of specific risk factors for TTI in Italy.

The possible exclusion of cats with a TTI risk profile from donor programmes contrasts with the difficulty of finding a sufficient number of adequate donors, as is confirmed by the enrolment of only 31 cats in more than 1 year. This makes the rejection of candidate donors very difficult, even if they have a TTI risk profile. Thus the application of a wide range of sensitive diagnostic tests should be proposed to verify the actual infectious status and to guarantee a sufficient number of adequate donors. American and European GLs recommend that donors with a high risk profile should undergo frequent or extensive TTI screening.[9,10]

Another possible action for increasing blood safety that is currently not recommended by GLs is a recruitment strategy that can identify populations of low TTI-risk donors. No general recruitment criteria are reported in veterinary medicine, and appropriate surveys should be taken to identify the best strategy. Laboratory-reared cats could be considered the ideal donors,[6] being negative for all the microorganisms, but enrolment of these animals is open to possible ethical issues.[10] A recent survey on hospitals with blood bank or transfusion services found that staff-owned cats are the donors enrolled most frequently, followed by colonies of feline donors and client-owned cats.[7] These sources of donors probably reflect different infection risks. Considering the central role

of the owner in managing health prophylaxis, determining the lifestyle of cats and choosing to donate blood, it is possible that informed and motivated owners could be a key point for safer blood donors that reduces the possible exposure to TTI risk factors.

Conclusions

Since the GLs recommend different protocols and can classify cats differently as eligible or ineligible for blood donation, the harmonization of recommendations would be advisable. This is especially important for the main TTI and for the choice of the most sensitive screening tests, with possible variations according to local epidemiological situations. These additional recommendations would improve the general level of transfusion blood safety. Screening costs and time may be reduced if appropriate tests are selected. Attention should be put to identify donors that were stray cats, with irregular or no ectoparasite treatments, travelling, or with outdoor access. The use of biomolecular method should be recommended, at least in the case of storage in blood banks, to identify proviral FeLV DNA, considering that rapid test is not definitively discriminatory, and haemoplasma DNA in blood collected for donation. Moreover, issues raised by FCoV seropositivity and possible presence of FCoV RNA in seronegative cats should be further considered.

Finally, appropriate recruitment protocols currently not considered in GLs, educational courses for owners, the possibility of establishing permanent groups of safe blood

donors and questionnaire-based risk profiles could improve the identification of suitable donors with low risk of harbouring TTI, reducing the necessity to perform extensive screening.

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Conflict of interest

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